

High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine

ROBERT J. WALL*, VERNON G. PURSEL*, AVI SHAMAY†, ROBERT A. MCKNIGHT†, CHRISTOPH W. PITTIUS†‡, AND LOTHAR HENNIGHAUSEN†§

*Reproduction Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705; and †Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT The whey acidic protein (WAP) is a major milk protein in mice, rats, and rabbits but has not been found in milk of livestock including swine. To determine whether mammary gland regulatory elements from the WAP gene function across species boundaries and whether it is possible to qualitatively alter milk protein composition, we introduced the mouse WAP gene into the genome of swine. Three lines of transgenic swine were analyzed, and mouse WAP was detected in milk from all lactating females at concentrations of about 1 g/liter; these levels are similar to those found in mouse milk. Expression of the corresponding RNA was specific to the mammary gland. Our results suggest that the molecular basis of mammary-specific gene expression is conserved between swine and mouse. In addition the WAP gene must share, with other milk protein genes, elements that target gene expression to the mammary gland. Mouse WAP accounted for about 3% of the total milk proteins in transgenic pigs, thus demonstrating that it is possible to produce high levels of a foreign protein in milk of farm animals.

Milk protein genes are transcribed in the mammary gland of lactating animals, and the encoded proteins are secreted in large quantities into milk. The whey acidic protein (WAP) is an abundant milk protein in mice (1, 2) but has not been found in swine or other livestock. Expression of the WAP gene is confined to the mammary gland (2, 3) and is under the control of steroid and peptide hormones as well as other developmental signals during pregnancy (4–6).

By targeting synthesis of foreign proteins to the mammary gland of transgenic animals, it should be possible to produce valuable proteins on a large scale in milk (7, 8). The combined properties of high activity and tissue-specificity make the murine WAP gene promoter a good candidate for targeting gene expression to the mammary gland. Towards this end we previously have expressed a hybrid gene containing regulatory elements from the mouse WAP gene and coding sequences from human tissue plasminogen activator in the mammary gland of transgenic mice (5, 6) and analyzed the protein in milk (5, 9). By characterizing the WAP gene, it may be possible to use its control elements to target expression of hybrid genes in farm animals. However, it is not known whether mammary regulatory elements are gene specific and whether they are functional across species boundaries. In addition, it is not known if the presence of a novel protein may adversely affect the physiology of the mammary gland. To address these questions we introduced the unmodified mouse WAP gene (10) into swine, which themselves do not contain a WAP gene, and analyzed expression of RNA and protein. With this approach, potential problems in interpreting expression data from hybrid genes would not be a factor. Also, potential deleterious physiological effects of a foreign

protein might be minimized because the target gene encodes a milk protein that would be confined primarily to the mammary gland.

Swine were chosen for these studies because they offer both economy in animal resources and time when compared to ruminantia as a transgenic animal model and because the questions being addressed did not require harvesting large quantities of milk that would be more easily obtained from dairy animals such as cows, goats, or sheep. The two primary constraints in any large animal transgenic project are the number of fertilized ova obtainable and the number of embryo recipients available. On average it is possible to recover 2–3 times more injectable ova per donor gilt than can be collected from a cow, doe, or ewe. The efficiency of producing expressing transgenic pigs or sheep per injected ovum is about 0.3% (calculated from refs. 11 and 12). Though a live-born-expressing transgenic calf has not been reported, a larger number of ova will probably be required to produce an expressing transgenic cow (13). Furthermore, because swine are polytocous, a recipient sow can carry 5 times as many fetuses as a cow, doe, or ewe. Additionally, the generation interval of swine is \approx 11 months, whereas that of goats is between 11 and 21 months and that of cattle at least 24 months. Considering all of these factors, the use of swine rather than cows, goats, or sheep requires one-sixth the number of animals, with results obtainable in less than half the time.

MATERIALS AND METHODS

Production of Transgenic Pigs. Ovulation control and egg recovery were performed as described (14). Briefly, the time of ovulation of sexually mature gilts was controlled by feeding 15 mg of Altrenogest (R-2267, 17-allyl-hydroxyestra-4,9,11-trien-3-one, Roussel-Uclaf) daily for 5–9 days, beginning on day 12 and ending on day 15 of the estrous cycle. Twenty-four hours after the last feeding of Altrenogest, each gilt was given 1000 to 2000 international units of pregnant mare's serum gonadotropin (PMSG) by subcutaneous injection, and 79 hr later each gilt was given an intramuscular injection of 500 international units of human chorionic gonadotropin (hCG). Estrus behavior was monitored, and embryo donor gilts were either bred with a fertile boar or were artificially inseminated with fresh semen twice during estrus.

Approximately 58–61 hr after the hCG injection (18–21 hours after the expected time of ovulation), the reproductive tracts of donor gilts were exposed by midventral laparotomy during general anesthesia. Ova were recovered by flushing 20 ml of Dulbecco's phosphate-buffered saline (15) from the uterotubal junction through the cannulated infundibular end

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Abbreviation: WAP, whey acidic protein.

‡Present address: Hoechst AG, Frankfurt, Federal Republic of Germany.

§To whom reprint requests should be addressed.

of each oviduct. Recovered ova were immediately transferred into BMOG buffer (16) prior to microinjection and maintained at 38°C.

Pig ova are optically opaque and, as a consequence, their nuclear structures are not visible. However, centrifuging ova at $\approx 15,000 \times g$ for 3–8 min displaces the opaque material in the cytoplasm, thereby allowing the nuclear structures to be visualized (14). Pig ova were centrifuged, and a pronucleus of one-celled ova or both nuclei of two-celled ova were injected with a TE solution (1 mM Tris-HCl/0.1 mM EDTA, pH 7.2) containing ≈ 2 ng of a 7.2-kilobase (kb) *EcoRI* fragment per μ l that contained the mouse WAP gene (10). The fragment contained the entire transcribed region with its four exons, three introns, and 2.6-kb 5' and 1.6-kb 3' flanking sequences. Microinjections were performed with the aid of differential interference contrast optics at 200-fold magnification, essentially as described for mouse ova (17).

Between 20 and 30 injected ova were deposited into the ampullar region of one oviduct of each recipient gilt whose reproduction cycle had been synchronized with Altrenogest (but not superovulated—i.e., not given PMSG) or whose estrous cycle naturally coincided with the desired stage. Some recipients also received 2–4 uninjected control ova to increase the likelihood of maintaining pregnancy in the event that a majority of the microinjected eggs failed to develop. Time between microinjection and embryo transfer was about 30 min.

To identify transgenic piglets, DNA from tail biopsies was prepared and analyzed for the mouse WAP gene by Southern blotting. Offspring in the F_1 generation were analyzed by the polymerase chain reaction by using primers specific to the WAP gene.

Analysis of Mouse WAP. Milk whey proteins were separated under denaturing conditions in sodium dodecyl sulfate (SDS)/16% polyacrylamide gels and either stained with Coomassie Blue or transferred to nitrocellulose filters. After transfer the membrane was incubated overnight in TBS (20 mM Tris-HCl, pH 7.5/500 mM NaCl) containing 3% gelatin and then was washed in TTBS (TBS containing 0.05% Tween 20). The membrane was then probed for 90 min with a 1:200 dilution of rabbit anti-WAP serum, followed by washing and incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 1% bovine serum albumin for 1 hr. The antibody-antigen complexes were stained with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$.

Isolation of RNA and Northern Blot Analysis. During necropsy, tissues were immediately placed in liquid nitrogen and stored at $-80^\circ C$, and total RNA was isolated (18). RNA samples containing 1 μ l of ethidium bromide solution (1 mg/ml) were electrophoresed in 1.5% agarose/formaldehyde gels. The gels were blotted onto GeneScreenPlus nylon membranes, which were then probed with a randomly primed labeled 450-base-pair (bp) cDNA fragment that spanned the mouse WAP coding region.

RESULTS

The Mouse WAP Gene in Transgenic Swine. Eight-hundred and fifty ova were recovered and microinjected, of which two-thirds were at the one-cell stage of development. The injected DNA contained 7.2 kb of the mouse WAP gene (see *Materials and Methods*, ref. 10). The microinjected ova along with 34 control ova were transferred into 29 recipient gilts. Twenty-two of the recipients carried their pregnancies to term, resulting in the birth of 189 pigs. DNA analysis of tail biopsies revealed that 5 (2 males and 3 females) of the piglets had incorporated the mouse WAP gene into their genomes. Approximately 1% of the injected ova resulted in transgenic

founders. From other transgenic pig projects using different gene constructs, the efficiency of producing founder pigs was similar (11). In this study one pig was stillborn and one died shortly after birth. Such deaths are not uncommon in the pig industry, where neonate mortality is in the range of 15–20%. Lines from the three surviving pigs were established, and offspring were analyzed. Male founder 1301 was bred to three nontransgenic females; 4 of 32 offspring were transgenic, suggesting that he was mosaic for the WAP gene. Transgenic mouse breeding studies have estimated that about 30% of transgenic founders are germ-line mosaics (19). Based on Southern blot analyses, this line contains ≈ 10 intact copies of the WAP gene in a head-to-tail arrangement at a single locus. Female founder 2202 carried ≈ 15 copies of the WAP gene. She was bred at 8 months of age; 4 of 9 offspring were transgenic. She was bred a second time and died of an unknown cause 4 days before anticipated parturition. The two transgenic daughters from her first litter were also bred, and after farrowing, milk and RNA were analyzed. Female founder 1302, carrying ≈ 10 copies of the WAP gene, was unsuccessfully bred three times. After the third failure, she was superovulated as a means of diagnosing the cause of her reproductive failures and to collect eggs if the cause did not involve ovarian dysfunction. Twenty-eight ova were recovered and transferred to two recipients. From these, 20 piglets were born of which 8 were transgenic. Apparently not all of female founder 1302's eggs had been recovered because she subsequently gave birth to 9 piglets, 5 of which were transgenic.

Secretion of Mouse WAP into Pig Milk. Expression of the WAP transgene in transgenic pigs was evaluated by both protein and RNA analyses. Milk from female founder 2202 and her daughter 5403, from two daughters (5511 and 5701) of male founder 1301, and from female founder 1302, was analyzed for the presence of mouse WAP. Milk proteins were separated in SDS/polyacrylamide gels and either stained with Coomassie blue or blotted onto nitrocellulose membranes and analyzed with anti-mouse WAP antibodies. WAP has a molecular mass of about 14 kDa (Fig. 1A, lane 8) and, at a concentration of about 2 mg per ml, constitutes the major

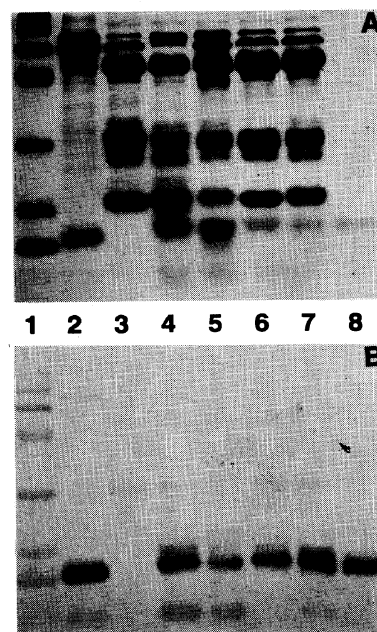


FIG. 1. Secretion of mouse WAP into milk of transgenic pigs. Milk proteins (20 μ g) were separated in SDS/polyacrylamide gels and either stained (A) or analyzed with rabbit anti-WAP antibodies (B). Lanes: 1, molecular mass markers (14, 18, 29, 45, 68, and 96 kDa); 2, total mouse whey proteins; 3–7, milk from nontransgenic pig (lane 3), pig 2202 (lane 4), pig 5403 (lane 5), pig 5701 (lane 6), and pig 5511 (lane 7); 8, 1 μ g of purified mouse WAP.

whey protein in mice (Fig. 1A, lane 2). A protein comigrating with mouse WAP was present in the milk of transgenic pigs (Fig. 1A, lanes 4–7) but not in milk from a nontransgenic control pig (Fig. 1A, lane 3). In addition, a 14-kDa protein in milk from transgenic, but not from nontransgenic, pigs reacted strongly with anti-mouse WAP antibodies (Fig. 1B). The lower molecular mass material reacting with anti-WAP antibodies probably reflects degradation products of the WAP. Taken together, this shows that the mouse WAP gene was expressed in transgenic pigs, and the encoded protein was secreted into the milk. The level of mouse WAP in the milk of each transgenic pig was determined in ELISA. By setting the level of WAP in mouse milk arbitrarily at 100%, animals 2202 and 5403 (line 2202) and animals 5701 and 5711 (line 1301) were shown to express WAP at about 100%, and female founder 1302, at about 50%. Thus, about 1–2 g of WAP was present per liter of pig milk.

WAP is secreted into mouse milk during the entire lactational period. To determine whether the expression in transgenic pigs paralleled this pattern, we analyzed WAP levels in the milk of founder female 1302 over a 4-week lactational period (Fig. 2). Whey samples were separated in SDS/polyacrylamide gels and either stained (Fig. 2A) or analyzed with anti-WAP antibodies (Fig. 2B). Constant levels of WAP were found over a 26-day period. This suggests that, at least over this period of time, the WAP transgene was coordinately regulated with other pig milk protein genes.

Expression of Mouse WAP RNA in Pigs. To correlate the level of WAP in milk with the corresponding RNA in mammary tissue, founder female 2202 was biopsied 11 days postpartum, and mammary RNA was analyzed with a mouse-specific WAP cDNA. An RNA of about 600 nucleotides hybridized with the WAP probe (Fig. 3, lanes b and c), confirming mouse WAP gene expression in the mammary glands of transgenic pigs. Furthermore, the RNA levels in pig 2202 and mouse were similar; this agrees with the WAP levels found in the milk. The WAP RNA in pig 2202 appeared to be

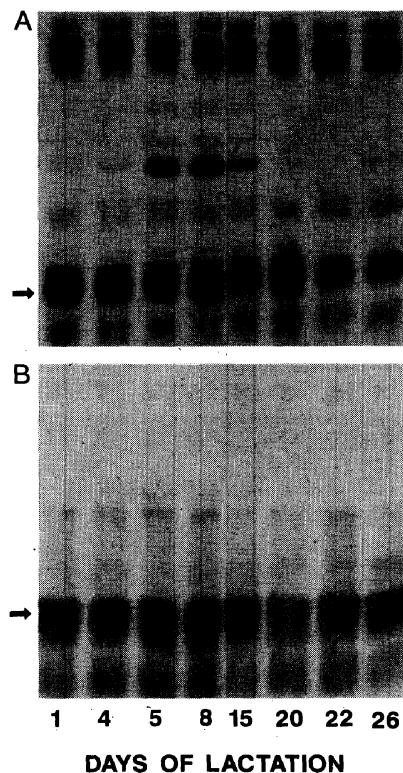


FIG. 2. Expression of mouse WAP during the lactational period of pig 1302. Milk samples were collected at various days after parturition as indicated, and whey fractions were prepared. Upon gel separation, samples were either stained (A) or analyzed with anti-WAP antibodies (B).

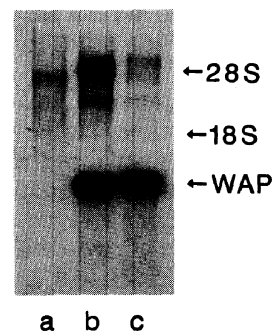


FIG. 3. Expression of mouse WAP RNA in transgenic pigs. Mammary RNAs (5 µg) from a lactating nontransgenic pig (lane a), founder pig 2202 (lane b), and a mouse (lane c) were separated in a formaldehyde gel, transferred to a nylon membrane, and analyzed with a cloned cDNA probe specific for mouse WAP RNA.

about 10–20 nucleotides shorter than its counterpart in mice (Fig. 3). Since the protein coding region was intact, the smaller size may be due to differences in polyadenylation. RNA from a nontransgenic pig did not hybridize with the WAP probe (Fig. 3, lane a), verifying the absence of an endogenous WAP RNA in the pig mammary gland.

In lactating mice the WAP gene is expressed almost exclusively in the mammary gland with levels in nonmammary tissues at least 4 orders of magnitude lower (5). To test whether the 7.2-kb WAP transgene contained elements for stringent tissue specificity observed in mice, we analyzed tissues from lactating pigs from lines 2202 and 1301 for the presence of WAP RNA (Fig. 4). To demonstrate potential WAP expression in nonmammary tissues, we exposed the RNA blot for 24 hr (Fig. 4a and c). The specificity of WAP hybridization and the quantity of WAP RNA in the mammary gland were assessed in a 30-min exposure (Fig. 4b). In animal 5701 (line 1301), WAP RNA was only found in the mammary gland (Fig. 4c) at a level similar to that seen in a 10-day lactating mouse. The sensitivity of the assay would have

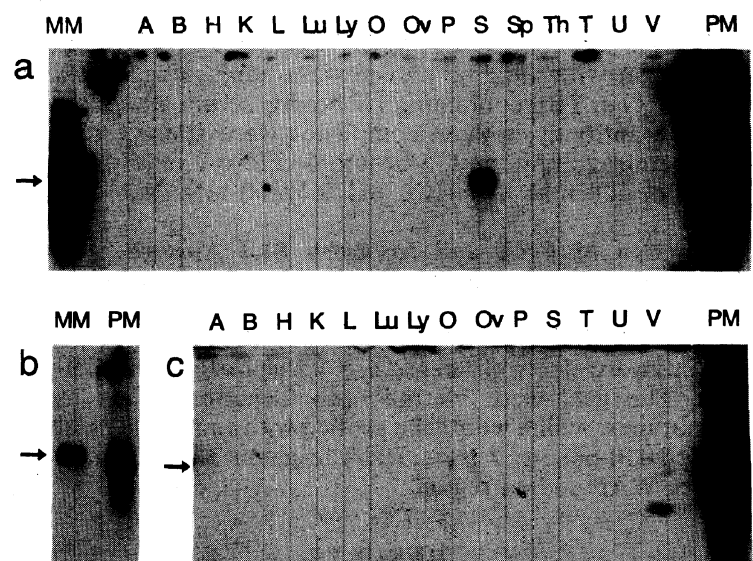


FIG. 4. Tissue distribution of WAP RNA in transgenic pigs. Pigs 5403 (a) and 5701 (c) were sacrificed, and RNA was prepared from several tissues. Upon separation in formaldehyde gels and transfer to nylon membranes, the RNA was analyzed with a probe specific for mouse WAP RNA. Lanes: MM, mouse mammary gland; PM, pig mammary gland; A, adrenals; B, brain; H, heart; K, kidney; L, liver; Lu, lung; Ly, lymph node; O, ovaries; Ov, oviduct; P, pituitary; S, salivary gland; Sp, spleen; Th, thymus; T, tongue; U, uterus; V, vulva. In a and c, 20 µg of total RNA was loaded in lanes with the exception of mouse mammary gland (lane MM), where 4 µg was loaded. (b) One-hour exposure of the MM and PM lanes of a. Arrows indicate the position of WAP RNA.

permitted detection of WAP RNA levels 1000-fold lower than that observed. The level of WAP RNA in animal 5403 (line 2202) was about 80% of that seen in mouse (Fig. 4a). The lower molecular mass band in the vulva RNA from animal 5701 was not reproducible and probably reflects a gel or blotting artifact. In animal 5403 WAP expression was detected in salivary gland, although at a level of only 1% of that seen in mammary tissue (Fig. 4a). Low-level expression in the salivary gland also has been described for other transgenes containing regulatory elements from milk protein genes (5, 20). Although the salivary gland and mammary gland have similar developmental patterns in that they require interaction between epithelial and mesenchymal tissue for proper duct formation to occur (21, 22), they are not considered closely related. In contrast, sebaceous glands have a common developmental origin to that of the mammary gland. However, no WAP transcripts were found in tissue taken from the vulva (Fig. 4), which is rich in sebaceous glands.

DISCUSSION

Three lines of transgenic swine containing the mouse WAP gene have been generated and analyzed. Although swine does not contain an endogenous WAP gene, its transcription machinery recognized the mouse WAP transgene in a tissue-specific manner, and mouse WAP was secreted into milk from founder swine as well as their offspring at levels similar to those seen in mouse milk. Thus, the molecular basis for mammary-specific gene expression is conserved between swine and mouse, and it can be suggested that the mouse WAP gene shares mammary regulatory elements with pig milk protein genes.

Expression levels of the mouse WAP genes in three lines of transgenic pigs described here and in three additional lines (unpublished data), which carry between 10 and 20 copies of the transgene, were consistently high and at a level comparable to the expression level of the endogenous gene in mice. Activity of the WAP gene in pigs appears to be relatively independent of the site of integration into host chromosomes and also independent of the gene copy number. In contrast, expression of the same 7.2-kb mouse WAP gene in transgenic mice was highly dependent on the integration site of the transgene (36). It remains to be determined whether the consistently high-level expression in transgenic pigs reflects special properties of the WAP gene, such as the presence of dominant transcription elements, or whether the pig genome provides a unique permissive environment for transgene expression. A host of other transgenic swine projects (23) argues against the latter explanation. Data from the sheep β -lactoglobulin gene (24), the rat WAP (25) and β -casein (26) genes, and several hybrid genes containing mammary regulatory elements (27–30) have shown that expression was influenced by the site of integration in transgenic mice. At a minimum the present study suggests that WAP gene regulation is different in mice and swine.

This study shows that it is feasible to synthesize and secrete a heterologous milk protein in the milk of farm animals at relatively high concentrations—i.e., more than 1 g/liter. Clark and colleagues had shown that hybrid genes containing regulatory elements from the sheep β -lactoglobulin gene are expressed in the mammary glands of transgenic sheep (31). However, the concentrations of the encoded proteins factor IX and α_1 -antitrypsin were only 25 μ g/liter and 5 mg/liter, respectively (31). With another transgene, this group produced human α_1 -antitrypsin in mouse milk at levels of more than 1 g/liter (20). Therefore, the ability of a transgene to be expressed in the mammary gland at high levels does not appear to be related to the nature of the encoded protein (milk protein versus foreign protein) but rather to the presence of appropriate transcription elements.

We are currently testing the ability of the mouse WAP gene promoter to control expression of non-WAP structural gene sequences in pigs.

The concentration of the transgene product produced in this study should be encouraging to those who envision using the mammary gland as a bioreactor for the production of foreign proteins as an economically viable alternative to existing tissue and microbial culture systems (7, 8). Swine produce about 10 kg of milk per day (32), and, based on the expression levels discussed here, it should be possible to produce the protein of interest at a rate of about 1 kg per lactational period of 7 weeks. Since the WAP gene promoter is active in pigs during their entire lactational period, this appears to be an achievable goal, and one sow could satisfy current world's demand of blood clotting factor IX. Alternatively, to the dairy industry, the modification of the composition of milk proteins themselves may be desirable so that overexpressing heterologous or endogenous milk proteins would result in novel milk products (33).

As with other expression systems, high activity of the transgene could have adverse effects on the physiology of the mammary gland. Pigs from two lines (1301 and 2202) were unable to sustain lactation. In contrast, lactation persisted normally in female founder 1302. This animal secreted less WAP into milk than those that abrogated lactation. Agalactia has not been observed in transgenic mice that secrete into their milk heterologous milk proteins (24, 34) or pharmacologically active proteins (20, 35) at levels similar to or exceeding those described here with swine. Experiments are in progress to determine whether the premature termination of lactation exhibited by some of the pigs is associated with mammary gene expression.

Note Added in Proof. We have generated transgenic mice with the 7.2-kb WAP transgene described in this paper and observed that some of the animals cannot maintain lactation (T. Burdon, R.J.W., and L.H., unpublished data).

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